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Exhibit B

Infection of Cynomolgus Monkeys with a Chimeric HIV-1/SIV$_{mac}$ Virus That Expresses the HIV-1 Envelope Glycoproteins

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Summary: Replication competent chimeric viruses that express the gag and pol proteins of SIV$_{mac}$ and the env proteins of HIV-1 were made. One such chimeric virus, SHIV-4, that expresses the *vif*, *vpx*, *vpr*, and *nef* regulatory genes of SIV and the *tat* and *rev* regulatory genes of HIV-1 replicated efficiently in cynomolgus monkeys. This model system can be used to evaluate the efficacy of anti-HIV-1 vaccines directed at the envelope glycoproteins, anti-HIV-1 envelope glycoprotein antiserum or monoclonal antibodies, and anti-HIV-1 drugs designed to inhibit the *tat*, *rev*, or *env* functions. Key Words: Chimeric virus—HIV/SIV—Cynomolgus monkey.

Human immunodeficiency virus type 1 (HIV-1) and human immunodeficiency virus type 2 (HIV-2) are the etiologic agents of AIDS in humans (1–3). These viruses are related to simian immunodeficiency viruses (SIV) that infect feral populations of sooty mangabeys, African green monkeys, and mandrills (for review, see refs. 4 and 5).

Development of a vaccine to prevent infection of HIV-1 requires a suitable animal model. The two animal models most commonly used, infection of chimpanzees with HIV-1 and infection of macaque monkeys with SIV, have limitations. HIV-1 does not replicate to high titers in chimpanzees, and infected chimpanzees do not develop immunodeficiency (6–10). Trials in chimpanzees are limited to a few animals as the species is endangered, available chimpanzees are few, and care is expensive.

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Rhesus and cynomolgus macaque monkeys infected with the macaque strain of SIV (SIV$_{mac}$) do produce high titers of virus and do develop an AIDS-like syndrome (11–13). Differences exist, however, in the immune responses to the HIV-1 and SIV$_{mac}$ envelope glycoproteins, which represent the principal targets for protective immunity (14–16). The major neutralizing antibodies in HIV-1–infected people are directed against two regions of the gp120 envelope glycoprotein. Antibodies against the HIV-1 third gp120 variable (V3) region have been shown to be protective (15). In contrast, the corresponding region of the SIV$_{mac}$ envelope glycoprotein does not exhibit sequence variation among isolates and is not a target for neutralizing antibodies in infected macaques (17,18). A second group of neutralizing antibodies in HIV-1–infected humans is directed against the conserved, discontinuous gp120 region that binds the CD4 viral receptor (19–29). These antibodies recognize HIV-1 gp120 regions distinct from those of the SIV$_{mac}$ gp120 glycoprotein recognized by antibodies from

infected macaques that neutralize multiple SIV strains (30–33). The differences between the antibodies that broadly neutralize HIV-1 and SIV strains are highlighted by the observation that such antibodies do not cross-neutralize (34).

To overcome these difficulties an attempt was made to create a chimeric virus between HIV-1 and SIV_{mac} that contains the HIV-1 envelope glycoproteins and is capable of replicating to high titers in macaque monkeys.

MATERIALS AND METHODS

Plasmid Constructions

The chimeric viruses were constructed using the infectious, pathogenic $SIV_{mac}239$ (*nef* open) virus (*gag*+, *pro*+, *pol*+, *vif*+, *vpx*+, *vpr*+, *tat*+ *rev*+, *env*+, *nef*+) (12,35) and the HXBc2 HIV-1 virus (*gag*+, *pro*+, *pol*+, *vif*+, *vpr*−, *tat*+, *rev*+, *vpu*−, *env*+, *nef*−) (36). All four chimeric viruses (designated SHIV) used in this study express the gag, pro, pol, vif, vpr, and nef proteins of $SIV_{mac}239$ (*nef* open) and the tat, rev, and env proteins of HIV-1 (HXBc2).

Each chimeric provirus clone was propagated in *E. coli* using two plasmids, one containing the 5′ half of the provirus and one containing the 3′ half of the provirus. The 5′ proviral clones, derived from the p239 SpSp 5′ plasmid (12), consisted entirely of sequences from the $SIV_{mac}239$ clone. The sequences from the 5′ cellular flanking sequences to the unique *Sph* I site in the $SIV_{mac}239$ genome were cloned into a pBS(+) plasmid (Stratagene) modified to contain a unique *Cla* I site in the polylinker region. This 5′ clone, which was used to generate the SHIV-1 and SHIV-2 chimeric viruses, contains the $SIV_{mac}239$ *tat* splice acceptor and *tat* initiation codon. Site-directed mutagenesis was used to create a modified 5′ clone in which the $SIV_{mac}239$ *tat* splice acceptor and *tat* initiation codon were modified (Fig. 1) This modified 5′ clone was used to generate the SHIV-3 and SHIV-4 chimeric viruses.

The 3′ proviral clones consisted of *tat*, *rev* and *env* sequences derived from the HXBc2 HIV-1 isolate and the *nef* and 3′ LTR sequences derived from the $SIV_{mac}239$ (*nef* open) isolate (35). In the $SIV_{mac}239$ (*nef* open) variant, the 93rd codon of *nef* is changed from a stop (TAA) to a Glu (GAA) codon, allowing production of a functional *nef* protein (35). The HIV/SIV_{mac} junction in the 3′ proviral clones was formed by ligating the HIV-1 and SIV_{mac} segments using the *Rsr* II site, which was created by site-directed mutagenesis in both the HIV-1 and $SIV_{mac}239$ (*nef* open) sequences.

To allow efficient ligation of the 5′ and 3′ proviral halves, a unique *Sph* I site was introduced by site-directed mutagenesis into the HIV-1 region upstream of the HIV-1 *tat* gene. This *Sph* I site was positioned such that the HIV-1 *tat* splice acceptor sequences would be either included in or excluded from the 3′ proviral clones. The 3′ clone that included the HIV-1 *tat* splice acceptor was used to generate the SHIV-1 and SHIV-3 chimeric viruses, while the 3′ clone lacking the HIV-1 *tat* splice acceptor was used to generate the SHIV-2 and SHIV-4 viruses (Fig. 1). Also, since the last few codons of the SIV_{mac} *vpr* gene are located 3′ to the natural *Sph* I site in the 5′ proviral clone, these codons were supplied by modification of the 3′ proviral clone near the introduced *Sph* I site. Thus, the *vpr* reading frame would be restored upon ligation of the 5′ and 3′ proviral clones at the *Sph* I site (Fig. 1).

Transfection of CEMx174 Cells with Chimeric Proviruses

For transfection, 5 μg of the 5′ and 3′ proviral clones were digested with *Sph* I and other restriction enzymes that recognize the flanking sequences (*Cla* I for the 5′ proviral clone and *Xho* I for the 3′ proviral clone). The fragments containing the 5′ and 3′ proviral sequences were ligated. The ligation reaction was then mixed with 3×10^6 CEMX174 cells suspended in 1 ml of serum-free RPMI 1640 and 500 μg/ml DEAE–dextran. The cell–DNA suspension was incubated at 37°C for 1 h, after which the cells were washed with serum-free medium and resuspended in 10 ml RPMI 1640 with 10% fetal calf serum.

Reverse Transcriptase Assays

Virus production in transfected or infected cultures was monitored every 3–4 days by reverse transcriptase assays as described, using 1.5 ml of cell-free supernatant (37). After removing supernatants for reverse transcriptase assays, cells were resuspended in a sufficient amount of fresh medium to maintain the cell density between 10^5 and 10^6 cells/ml.

Infection of Cultured Monkey PBMCs

Typically, $2–4 \times 10^7$ peripheral blood mononuclear cells (PBMCs) were isolated from 15–30 ml whole blood from cynomolgus monkeys. Cells were isolated using Ficoll–Paque (Pharmacia) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum and either phytohemagglutinin (PHA-C) (Boehringer-Mannheim) or concanavalin A (Con A, type IV, Sigma) at 5 μg/ml. Three to 5 days following PHA-C or Con A stimulation, the cells were washed and resuspended in RPMI 1640 with 10% fetal calf serum and 10 U/ml interleukin-2 (human recombinant, Boehringer-Mannheim). Two days later, PBMCs were infected with 1×10^5 reverse transcriptase units of virus derived from transfected CEMx174 cells. Three days after infection, PBMCs were washed and resuspended in fresh medium. Reverse transcriptase measurements in cell supernatants were made on days 4, 6, 9, and 13 following infection.

Preparation of Virus Stocks and $TCID_{50}$ Determination

Virus stocks for animal inoculation were prepared in cynomolgus monkey PBMCs and frozen as cell-free supernatants without additives at −70°C. The virus titer was determined by incubating 100 μl of thawed stocks, either undiluted or as 10-fold serial dilutions, in quadruplicate with 1×10^5 CEMx174 cells in 1 ml of medium. When cultures became confluent, cells were diluted 1/10. The wells were scored for the presence of syncytia after 2 weeks, and the 50% tissue culture infectious dose ($TCID_{50}$) in the virus stock calculated as described (38).

Immunoprecipitation of Infected Cultures

Approximately 2×10^6 CEMx174 cells were infected with HIV-1 (HXBc2 strain), $SIV_{mac}239$ (*nef* open), or chimeric viruses. The cultures were labeled overnight with [^{35}S]cysteine 1–2 days prior to the peak of syncytium formation, and cell lysates were precipitated either with serum from a HIV-1-infected AIDS patient or from a SIV_{mac}-infected rhesus macaque as described (30).

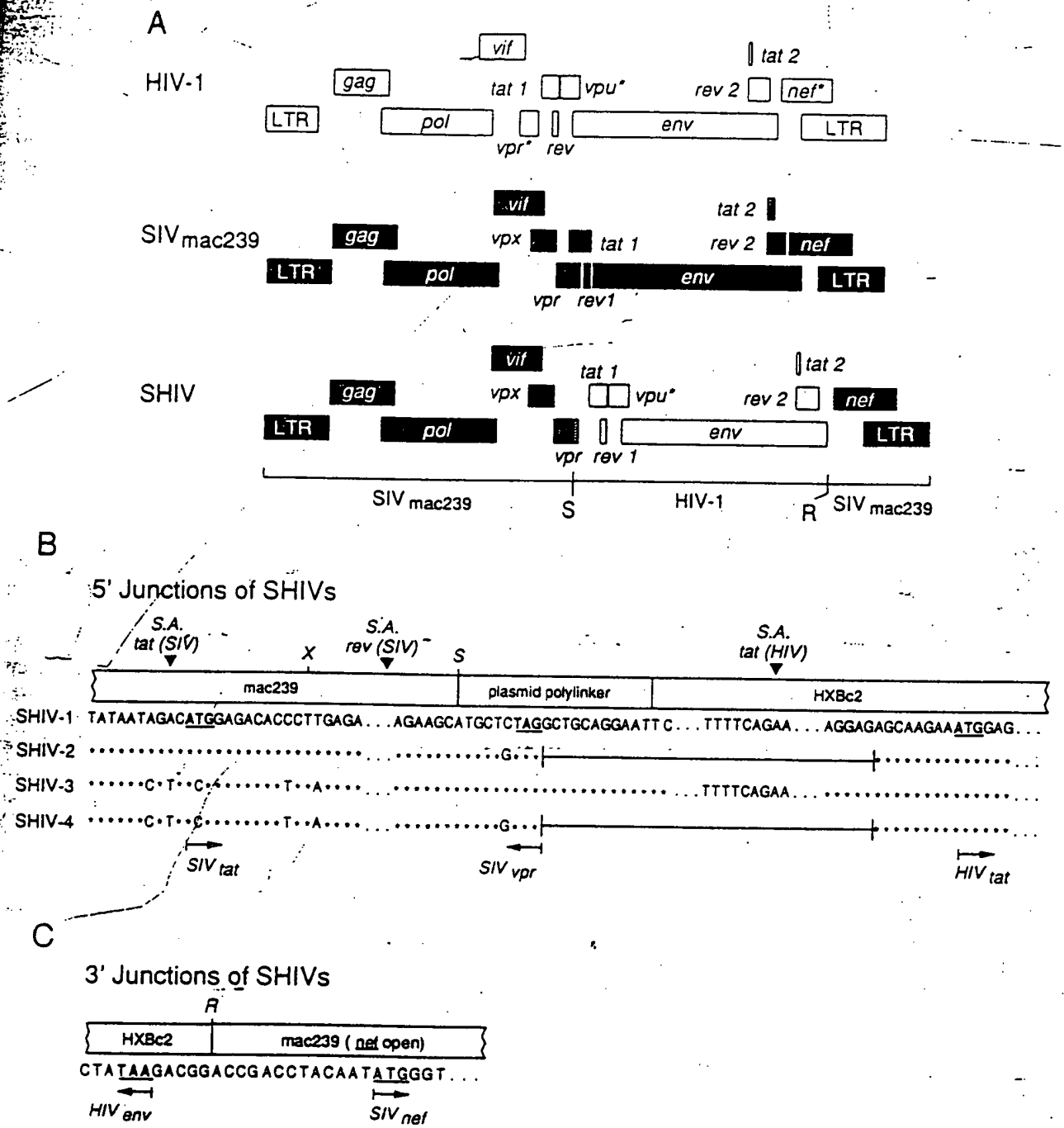

FIG. 1. Structure of the chimeric viruses. **A:** The genetic organization of the HIV-1 (HXBc2), SIV$_{mac}$239 (*nef* open), or SHIV chimeric viruses is shown, with HIV-1- or SIV$_{mac}$-specific sequences designated as white or black boxes, respectively. Genes that are defective in the strains utilized are denoted with an asterisk. The 5' SIV$_{mac}$/HIV-1 junction at the *Sph* I site (S) and the 3' HIV-1/SIV$_{mac}$ junction at the *Rsr II* site (R) are shown. The stippled 3' end of *vpr* of the SHIV virus represents sequences derived from the HIV-1 portion of the chimera that reconstitute the SIV$_{mac}$239 *vpr*. **B:** The details of the 5' SIV$_{mac}$/HIV-1 junction near the *Sph* I site (S) are shown for each of the SHIV chimeric viruses. The positions of the splice acceptors (S.A.) for the SIV$_{mac}$ *tat* and *rev* messages and for the HIV-1 *tat* message are shown above the figure, with the SIV$_{mac}$ *tat* initiation codon, SIV$_{mac}$ *vpr* stop codon and HIV-1 *tat* initation codon underlined and labeled wjth arrows below. The asterisks denote sequence identity and the dots represent sequences not shown. The horizontal bars represent sequence deletions. The X marks the position of an *Xba* I site in the SHIV-3 and SHIV-4 sequences. **C:** The details of the 3' HIV-1/SIV$_{mac}$239 (*nef* open) junction near the *Rsr* II site (R) are shown. The stop codon for the HIV-1 *env* and the initiation codon for the SIV$_{mac}$ *nef* are underlined and labeled with arrows.

Inoculation of Cynomolgus Monkeys with Chimeric Virus

Two male and two female cynomolgus monkeys (*M. fascicularis*) were inoculated intravenously with 1 ml of virus stock containing 7×10^3 $TCID_{50}$ of the SHIV-4 chimeric virus.

Virus Isolation from Inoculated Cynomolgus Monkeys

At 2 and 4 weeks following inoculation of cynomolgus monkeys, CD8-depleted, Con A-stimulated PBMCs were cultured from each animal and the level of SIV_{mac} gag p27 antigen in culture supernatants assessed as described (39). Culture supernatants positive for viral antigen were used to infect CEMx174 cells, which were labeled and used for immunoprecipitation as described above.

RESULTS

Chimeric Viruses

The goal of these studies was to create a replication-competent SIV/HIV-1 hybrid virus that expresses the HIV-1 envelope glycoproteins. The sequences used for the construction were derived from the pHXBc2 DNA, a clone prepared from the IIIB strain of HIV-1 (36), and the p239 SpSp 5′ and p239 SpE3′/nef-open plasmids derived from the $SIV_{mac}239$ strain of virus (12,35). The $SIV_{mac}239$ viral DNA was selected to be one of the parents for the recombinant virus as injection of cynomolgus or rhesus monkeys with either purified viral DNA or virus derived from this DNA resulted in both high levels of viremia and an AIDS-like disease (12,35, 40).

Construction of the appropriate chimeric molecules was complicated by significant differences in the regulatory genes of the two viruses as well as the complex genetic organization of the primate immunodeficiency viruses (5,41,42). Both HIV-1 and SIV_{mac} encode the regulatory genes *vif, vpr, tat, rev,* and *nef*. The regulatory gene *vpu* is specified only by HIV-1 (43–48), whereas *vpx* is found only in HIV-2 or SIV (49–51). The strategy used for construction of chimeric viruses was to replace the *tat, rev,* and *env* sequences of $SIV_{mac}239$ by the corresponding sequences of HXB2. The resultant viruses should contain the LTR *gag, pol, vif, vpx, vpr* and *nef* of SIV_{mac} and *tat, rev,* and *env* of HIV-1.

The original chimeric virus, designated SHIV-1 (SIV-HIV-chimeric virus-1), contains two *tat* splice acceptor sequences. The 5′ *tat* splice acceptor sequence is of SIV_{mac} origin whereas the 3′ *tat* acceptor sequence is derived from HIV-1 sequences. To minimize the possibility that the presence of two closely spaced splice acceptor sites might interfere with one another, derivatives of SHIV-1 were made that contain only the SIV_{mac} splice acceptor site (SHIV-2), only the HIV-1 splice acceptor site (SHIV-3) or neither splice acceptor site (SHIV-4) (Fig. 1). In the virus that lacks both *tat* splice acceptors, it is likely that the remaining SIV_{mac} *rev* acceptor substitutes for the *tat* acceptor.

Replication of Chimeric Viruses in Culture

The parental $SIV_{mac}239$ virus replicates well in the human $CD4^+$ B/T cell hybrid line CEMx174 (52). To determine whether chimeric SHIV DNAs produce infectious virus, CEMx174 cells were transfected with the parental $SIV_{mac}239$ as well as SHIV recombinant DNAs. Virus replication was monitored by measurement of the amount of the viral DNA polymerase (reverse transcriptase) released into the culture medium.

The data of Fig. 2 show that virus is produced from cultures that are transfected with all five DNAs. However, significant differences in the rate of appearance of reverse transcriptase in the medium were noted using different DNAs. Significant virus replication was evident by 9 days post-transfection in cultures treated with either the parental $SIV_{mac}239$ DNA or the SHIV-2 or SHIV-4 DNAs (Fig. 2A). Detectable levels of reverse transcriptase were not present in the cultures transfected with SHIV-1 or SHIV-3 DNAs until day 13 post-transfection (Fig. 2B). The relative delay in appearance of virus in the supernatant of cultures transfected with SHIV-1 or SHIV-3 DNAs as compared to those transfected with $SIV_{mac}239$, SHIV-2 or SHIV-4 DNAs was observed in several independent experiments. Despite this reproducible delay, the rates of replication of all four chimeric viruses were indistinguishable when similar amounts of virus harvested from the supernatant fluids of the transfected cultures were used to reinfect CEMx174 cells (data not shown).

The ability of SHIV-2 and SHIV-4 viruses to initiate infection in primary PBMCs derived from cynomolgus monkeys was examined. For these experiments the $SIV_{mac}239$, SHIV-2, and SHIV-4 viruses harvested from the supernatant fluids of transfected CEMx174 cells were incubated with PHA-1- or Con A-activated monkey PBMCs. Three days after infection with these viruses, the PBMCs were washed and resuspended in fresh medium. Virus replication

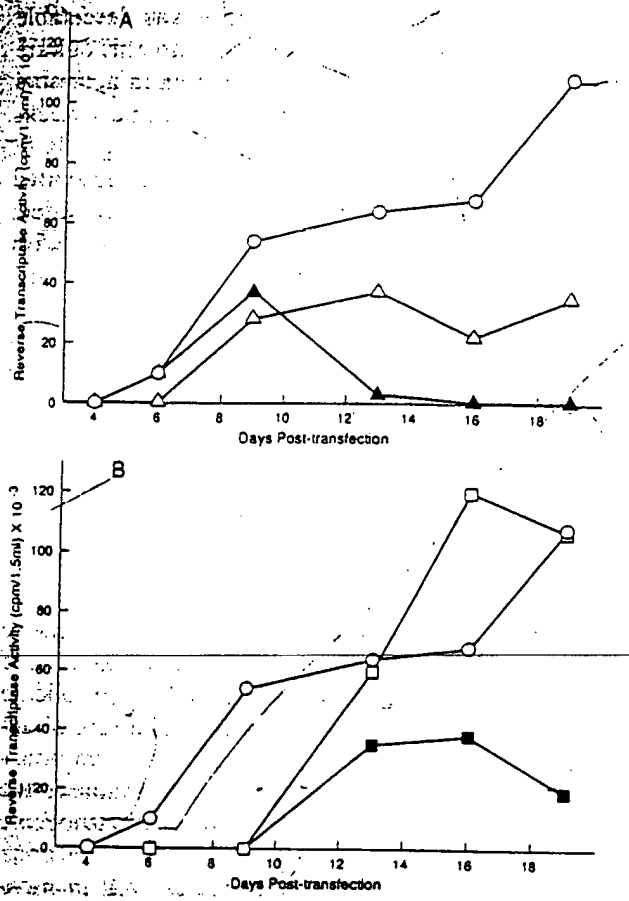

FIG. 2. Replication of chimeric viruses in CEMx174 lymphocytes. The reverse transcriptase activity in the supernatants of CEMx174 cells that had been transfected with proviral DNA of SIV$_{mac}$239 (*nef* open) (○) or chimeric viruses is shown. A: The CEMx174 cells were transfected with SHIV-2 (▲) or SHIV-4 (△) DNA. B: The CEMx174 cells were transfected with SHIV-1 (■) or SHIV-3 (□) DNA.

was measured by detection of reverse transcriptase activity in culture supernatant fluids.

The data of Table 1 show that all three viruses replicated well in cultures of PBMCs derived from cynomolgus monkeys. The rate of replication and amount of virus produced upon infection of the monkey PBMCs with either the SHIV-2 or SHIV-4 virus was similar to that obtained upon infection of the culture with SIV$_{mac}$239.

TABLE 1. *Reverse transcriptase activity (cpm/1.5 ml × 10^{-3}) in supernatants of cynomolgus monkey PBMCs*

Virus	Days after infection			
	4	6	9	13
SIV$_{mac}$239 (*nef*-open)	33	45	30	61
SHIV-2	96	82	41	28
SHIV-4	57	76	17	34

Chimeric Nature of the Recombinant Viruses

The SHIV chimeras should produce gag and pol products of SIV$_{mac}$ and env proteins of HIV-1. The viral gag proteins of HIV-1 and SIV$_{mac}$239 can be distinguished by mobility differences on SDS–polyacrylamide gels, following precipitation with sera from HIV-1-infected humans or SIV$_{mac}$-infected monkeys. Such sera contain antibodies that cross-react with gag but not with env proteins (53). To determine whether the SHIV viruses that replicate in cynomolgus monkey PBMCs express both SIV$_{mac}$ and HIV-1 proteins, viruses harvested from the supernatant fluids of infected PBMC cultures were used to infect CEMx174 cells. As controls, CEMx174 cells were infected with SIV$_{mac}$239 (*nef* open) and HIV-1 (HXBc2) viruses. The infected cells were labeled with [^{35}S]cysteine, lysed, and the viral proteins precipitated with serum from a HIV-1-infected AIDS patient or serum from a SIV$_{mac}$-infected macaque. The precipitates were analyzed on SDS–polyacrylamide gels.

The data of Fig. 3A show that, as expected, both the human and monkey sera recognize gag proteins of the parental HIV-1 and SIV viruses. These proteins can be distinguished from one another by electrophoretic mobility of both the capsid proteins (HIV-1 p24 and SIV$_{mac}$ p27) and the gag precursor proteins (HIV-1 p55 and SIV$_{mac}$ p58). The HIV-1 serum recognizes the gp160 and gp120 env glycoproteins present in CEMx174 cells infected with HIV-1 but not the env proteins of cells infected with SIV$_{mac}$239. The anti-SIV$_{mac}$ serum recognizes the gp160 and gp130 env proteins present in cells infected with SIV$_{mac}$239 but not with the HIV-1 virus.

In these experiments the gag proteins present in cells infected with the SHIV-4 virus exhibited the electrophoretic mobility characteristic of SIV$_{mac}$ capsid proteins. The env proteins of these extracts were recognized by the anti-HIV-1 but not the anti-SIV$_{mac}$ serum. The electrophoretic mobilities of the env proteins present in cells infected with the SHIV-4 virus corresponded to those expected for the envelope glycoproteins of HIV-1. These experiments confirm that the SHIV-4 virus is chimeric and produces the gag proteins of SIV$_{mac}$ and the env proteins of HIV-1.

Infection of Cynomolgus Monkeys

The ability of one of the chimeric viruses, SHIV-4, to replicate in cynomolgus monkeys was exam-

Shibata et al. (57) have also constructed a SIV$_{mac}$/HIV-1 chimeric virus that expresses the HIV-1 envelope glycoproteins. This virus is defective for both *vpr* and *nef*. The chimera described by Shibata et al. replicates in cultured monkey PBMCs. However, the rate of replication of this virus appears to be slower than that of the SIV$_{mac}$ parental virus (57). The absence of *vpr* and *nef* and suboptimal splicing patterns may account, at least in part, for this delay.

The presence of the HIV-1 *tat* splice acceptor near the 5′ SIV$_{mac}$/HIV-1 junction slowed the appearance of virus in cells transfected with the SHIV-1 or SHIV-3 DNA. It is likely that the presence of the HIV-1 *tat* splice acceptor results in inefficient expression of viral genes. The ability of virus harvested from CEMx174 cells transfected with proviruses containing the HIV-1 *tat* acceptor sequence to initiate efficient reinfection of CEMx174 cells probably indicates that selection for optimal splicing occurs rapidly in culture.

The experimental system described here (the infection of cynomolgus monkeys with the SHIV-4 virus) should provide a valuable model for study of the efficacy of anti-HIV-1 vaccines. The ability of such vaccines to induce protective immune responses in monkeys to infection by SHIV-4 should provide an indication of efficacy against viruses with HIV-1 envelope glycoproteins. The model can also be used to evaluate the ability of polyclonal and monoclonal antibodies to inhibit HIV-1 envelope function in animals. Therapeutics designed to inhibit the HIV-1 *tat*, *rev*, or *env* functions can also be evaluated in this model system.

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